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Growth of injected melanoma cells is suppressed by whole body exposure to specific spatial-temporal configurations of weak intensity magnetic fields

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Abstract

Purpose: To measure the effect of exposure to a specific spatial-temporal, hysiologically-patterned electromagnetic field presented using different geometric configurations on the growth of experimental tumours in mice.

Methods: C57b male mice were inoculated subcutaneously with B16-BL6 melanoma cells in two blocks of experiments separated by six months (to control for the effects of geomagnetic field). The mice were exposed to the same time-varying electromagnetic field nightly for 3 h in one of six spatial configurations or two control conditions and tumour growth assessed.

Results: Mice exposed to the field that was rotated through the three spatial dimensions and through all three planes every 2 sec did not grow tumours after 38 days. However, the mice in the sham-field and reference controls showed massive tumours after 38 days. Tumour growth was also affected by the intensity of the field, with mice exposed to a weak intensity field (1–5 nT) forming smaller tumours than mice exposed to sham or stronger, high intensity (2–5 μT) fields. Immunochemistry of tumours from those mice exposed to the different intensity fields suggested that alterations in leukocyte infiltration or vascularisation could contribute to the differences in tumour growth.

Conclusions: Exposure to specific spatial-temporal regulated electromagnetic field configurations had potent effects on the growth of experimental tumours in mice.

Keywords: *frequency-modulated, magnetic fields, mice, melanoma, nocturnal exposures, spatial configurations*

Introduction

The effect of electromagnetic fields on cancer rates in human populations is very controversial. Conflicting results from different studies suggests a complex relationship between electromagnetic fields and cell signalling. It has been suggested that the effectiveness of electromagnetic fields on biological systems is dependent on waveform pattern of the fields such as frequency, duration, wave shape, and relative orientation of the fields (Liboff et al. 1987, Blackman et al. 1990, Pilla et al. 1999). Exposure to low frequency, time-varying electromagnetic fields with simple or temporally symmetrical (sine-wave or square-wave) patterns have been shown to affect signalling pathways in some cells (Simko and Mattsson 2004). For example, exposure of MCF-7, HL-60, or 3Y1 cells to 60 Hz, 100 μT (1 Gauss) electromagnetic fields for 10–30 min activated extracellular-related kinase (ERK) phosphorylation to the same extent as treatment with 0.1–0.5 ng/ml phorbol ester (Jin et al. 2000, Nie and Henderson 2003). Exposure of MCF-7 cells to 50 Hz sinusoidal fields at only 1.2 μT for three days blocked the melatonin-induced inhibition of cAMP accumulation but not GTPase or adenylyl cyclase activities (Ishido et al. 2001). Some studies have shown that exposure of breast cancer cells, fibroblasts, keratinocytes, or peripheral blood mononuclear cells to 50 or 60 Hz electromagnetic fields activates the expression of specific genes including estrogen receptor cofactors, growth response genes, chemokines, and

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cytokines (Reale et al. 2006, Li et al. 2007, Girgert et al. 2008, Kaszuba-Zwoinska et al. 2008, Vianale et al. 2008).

The observation that some studies (Coulton et al. 2004, Alfieri et al. 2006, Antonini et al. 2006, Bernardini et al. 2007) do not show changes in gene expression suggests that specific properties of the time-varying field or of the target cells may be important for inducing biological effects. It has been suggested that exposure of HeLa cells to 60 Hz, 8 μT electromagnetic fields can activate a specific 'electromagnetic response element' in the heat shock protein-70 (HSP70) promoter (Lin et al. 2001). This ability of electromagnetic fields to induce HSP70 has been linked to improved myocardial function following ischemia in rats (George et al. 2008). Low frequency electromagnetic fields have also been suggested as a means to activate immune responses (Simko and Mattsson 2004) and exposure of mice to electromagnetic fields can promote tumour necrosis factor-α (TNF-α) expression in the mouse spleen (Yamaguchi et al. 2006). Furthermore, Yamaguchi et al. (2006) found that mice injected with murine B16-BL6 melanoma cells and then exposed to electromagnetic fields for only 80 s per day for 17 days using a 0.25 T magnetic field at 25 Hz (pulse width = 238 μs) showed a significant reduction in tumour weight.

Melanomas are a particularly aggressive form of tumour. The prognosis of a patient with an invasive, vascularised tumour is poor following treatment with chemotherapies or surgery (Lang 2002). Therefore, alternate modes of treatment that alter melanoma invasion and growth could be very advantageous. It has been suggested that the biological efficacy of electromagnetic fields, determined by their temporal patterns, may provide a new mode of treatment (Martin et al. 2004).

Unlike chemical treatments that are strongly dependent on the vascular system for delivery, extremely low frequency magnetic fields directly penetrate tissue. Furthermore, the magnetic fields that are able to simulate natural biogenic patterns are effective at relatively low (nT to μT) intensities (Persinger 2003). Several different studies have shown that exposure to low frequency electromagnetic fields for a few hours every day can inhibit cell growth including inhibition of goblet cells in rat eye conjunctiva (Keklikci et al. 2008), promote differentiation in epithelial cell lines (Lisi et al. 2006), and promote apoptosis in a subset of cells in the rat bowel (Kaszuba-Zwoinska et al. 2005). Other studies have shown exposure to magnetic fields can enhance the growth of chondrocytes (Nicolin et al. 2007) and osteoblasts (Zhang et al. 2007) and can alter the expression of metabolic and cell matrix genes in chondrocytes and mesenchymal precursors in a manner dependent on the type of electromagnetic field applied (Walther et al. 2007).

We hypothesised that electromagnetic fields with an optimal combination of spatial complexity and temporal pattern might be able to conspicuously suppress B16-BL6 melanoma cell growth even at weaker field intensities. In previous studies we had found that 1 μT magnetic fields whose applications were rotated successively through the three spatial dimensions produced powerful affects on learning if the pattern was chosen to simulate the brain's natural patterns (McKay et al. 2000). We reasoned that an extremely low frequency-modulated magnetic field pattern rotated through the three spatial axes during nocturnal (red light) periods (Olcese 1990) would be sufficient to interfere with the normal growth signalling pathways in B16-BL6 melanoma cells and thus diminish their ability to form tumours.

Materials and methods

Subjects

A total of 65 male C57b mice (90–120 days old) were used in these experiments. The experiment was performed in two separate blocks separated by six months to control for potential effects of the endogenous geomagnetic field: Block one contained 32 mice and block 2 contained 33 mice (one group contained five mice, see below). Each mouse was injected subcutaneously in the right hind flank with 2×10^5 B16-BL6 cells. The mice in each block were injected with an aliquot from the same B16-BL6 cell preparation and then groups of four mice were randomly assigned to different treatment groups and housed together in standard plastic cages. The mice had been habituated to the colony environment for four weeks after arrival from the breeders (Charles River, Pointe-Claire, QB, Canada). The light:dark cycle was 12:12 with the onset at 08:00 h local time and at a constant temperature of $20 \pm 1^{\circ}$C. Corncob bedding was changed once per week. All procedures were reviewed and approved by the Laurentian University Animal Care Committee (Protocol #2005-08-03) and the mice were treated according to the guidelines of the Canadian Council on Animal Care.

Procedure

Beginning on the day of cancer cell injection, the mice were exposed to two control and six different magnetic field configurations (see below) in red light between 21 h and midnight (3 h) every day for 38 days. Each treatment group of four mice (for each block) were transferred from their home cages,

placed into normal ($20 \times 30 \times 20$ cm) plastic cages situated within apparatus to generate the different magnetic field configurations, and then immediately returned to their home cages. The exposure volumes for each apparatus were comparable. The health and status of the mice were monitored twice per day (02:00 and 14:00 h). Tumours were visually apparent approximately two weeks after injection of the cells and then grew quickly and achieved approximately the same mass and volume within the various control groups. After 38 days, the mice were sacrificed using carbon dioxide, the tumours measured and isolated, and the tissues from three mice from each cage processed for histology (tumours from the fourth mouse were set aside for future RNA isolation).

This day was selected according to endpoints agreed upon by the university Animal Care Committee and the extensive size of the subcutaneous tumours within the control groups. To document the size and presence of tumours in each mouse, the skin over the back was removed for inspection around the injected site and photographs taken. Post-mortem inspection of the viscera was performed to ensure there had been no obvious metastasis. The presence or absence of tumours was determined for each group and compared by Chi-squared analyses using SPSS computer software on a VAX 2000 computer.

Equipment for magnetic field generation

In all experiments a specific frequency modulated magnetic field pattern, called the Thomas field (Figure 1), was used. The Thomas field was previously shown to increase analgesic effects (Thomas et al. 1997) and to promote seizure production in rats (Persinger and Belanger-Chellew 1999). The magnetic field was generated from solenoids using a direct electric current created by

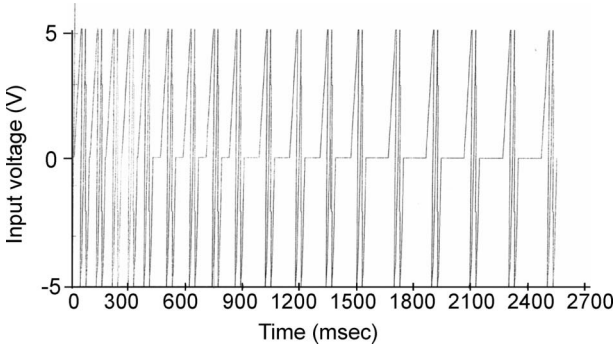

Figure 1. The Thomas EMF is a frequency-modulated pattern composed of 849 points each programmed for 3 msec using a digital-to-analog device. The specific temporal characteristics of the pattern are described in the text.

a custom-constructed digital-to-analogue (DAC) device that converted numbers between 1 and 256 to voltages between -5 V and $+5$ V at each point (Martin et al. 2004). The Complex© software, programmed on an IBM XT computer, was used to set the duration and voltage at each point. The Thomas pattern is composed of 849 points each programmed for 3 ms (each cycle lasting 2.55 s).

The Thomas pattern is made of 18 doublet peaks: Each peak lasts 15 ms, shows a gradual increase, and then a maximal excursion (-5 V to $+5$ V) over 6 ms. For each cycle, the doublet peak was repeated five times (25 Hz), an additional time delay of 30 ms was inserted between the doublet peaks and repeated four times, a time delay of 90 ms was inserted between the doublet peaks and repeated another four times, and a time delay of 120 ms was inserted between the doublet peaks and repeated a final five times (6 Hz). The entire cycle was repeated after a 3 ms delay for the 3 h exposure period.

The mice were exposed to the Thomas pattern magnetic field (or control conditions) using different apparatus to alter the application geometry. It was previously shown that rats exposed to an environment with an homogenous field showed lower levels of analgesia compared to rats exposed to a heterogenous field (Martin and Persinger 2003) and therefore the effects of different application geometries on tumour growth were measured. The apparatus in this study was used to generate eight different exposure conditions and controls as described in Table I. The cylindrical field, 5 μT at the central axis (condition 2), was generated between two 25×1 cm solenoids, created by 1050 turns of insulated wire around an iron nail, separated by 20 cm (Martin et al. 2004). A homogenous field of 5 μT through the exposure area (condition 3), was generated using two 30 ohm Helmholtz coils, placed 30 cm apart, each constructed by wrapping a 39×39 cm box with 305 m of 3 American Wire Gauge (AWG, Belden #9978, Belden, Cobourg, ON, Canada) wire in a single layer 18 cm wide.

The Helmholtz coils had an internal static geomagnetic field attenuated to approximately 35 μT by shielding. The opposing double coils were created by wrapping two 112×125 cm plastic boxes with 72 turns of 30 AWG wire in a single layer of 13 cm wide, placed 2 m apart; the high intensity field (2–5 μT) was generated in the housing area closest to the activated coil (condition 5) while the low intensity field (1–5 nT) was generated in a separate, adjacent housing area furthest from the activated coil (condition 6), while the attenuated field (condition 4) was generated in the apparatus when the coils were not activated. Furthermore, the field between two opposing double coils (conditions 4, 5 and 6) was presented in a region where two stainless steel

Table I. Electromagnetic field exposure conditions used.

Exposure condition		Treatment conditions	Field strength	References
1	Control	Never touched except to clean cages		
2	Cylindrical field	Heterogenous field generated by one-dimensional (single plane) solenoids	5 μT at the centre	Martin et al. 2004
3	Helmholtz coil	Spatially homogenous field over the exposure area generated between Helmholz coils	5 μT	Martin et al. 2004; Persinger et al. 2005
4	Attenuated field	Two opposing double coils where the local geomagnetic field had been attenuated to 5–15 μT		St-Pierre et al. 1998
5	High intensity field	The housing area placed nearest the two opposing double coils	2–5 μT	Martin et al. 2004
6	Low intensity field	The housing area placed furthest from the two opposing double coils	1–5 nT	Martin et al. 2004
7	Spatially rotating field	The frequency modulated field was generated successively for 0.5 s each through opposing 50 ohm solenoids with the X, Y, and Z planes and then all planes (X + Y + Z) simultaneously before the sequence repeated	2–5 μT	McKay et al. 2000
8	Sham field	Animals were transferred to the spatially rotating apparatus with no induced field		

Further details for the construction of the individual apparatus are available in the text and in the indicated references. All magnetic field exposure conditions (2 through 7) used the Thomas signal, which is a frequency-modulated field.

shelves provided magnetic field shielding (St-Pierre et al. 1998) of the local geomagnetic field to 5–15 μT (compared to the normal geomagnetic field of approximately 50 μT). This was done in order to meet the criterion for the cyclotron resonance frequency of calcium ($f_C = 7$ Hz; Lisi et al. 2006) when using an apparatus that produces an applied field with primary frequency modulated patterns of 7.6 Hz for 10 μT to 11.4 Hz and 15 μT, where $f_C = Boq/2\pi m$ (Bo is the component of the static geomagnetic field and q and m are the charge and mass of the ion, respectively) (Liboff et al. 1987). Variants of cyclotron resonance solutions are popular models (Lednev 1991, Blanchard and Blackman 1994) to understand the mechanisms by which weak magnetic fields can influence cell systems. Because of the premature death of four mice exposed to the low intensity double-coil fields (1 in one block and 3 in the second) the second experimental block contained a group of five mice and only 5/9 mice remained for that condition. The spatially rotating magnetic field (condition 7) was generated by 3 pair of 50 ohm solenoids (28P-I-24VDC, Guardian Electric Manufacturing, Woodstock, IL, USA, each fitted with a 20×0.75 cm iron rod) where each solenoid was placed at the centre of each face of a $30 \times 30 \times 30$ cm cube. The frequency-modulated field (2–5 μT) was generated successively for 0.5 s through opposing solenoids within the X, Y, and Z planes, and then all planes (X + Y + Z) simultaneously for 0.5 s (0.5 Hz) before the sequence began again (McKay et al. 2000). In all cases, the strengths of the fields were determined experimentally using a MEDA FM 300 vector magnetometer (Macintyre Electronic Design Associates, Dulles, VA, USA).

Histology

On the basis of visual differences in tumour size, the tissues were fixed in ethanol-formalinacetic acid. [The tumours excised from mice exposed to the low intensity field (condition 6) were approximately half the size of the tumours in mice exposed to the sham field (condition 8) or the high intensity field (condition 5).] After processing, the tissues were embedded in paraffin, cut at 6 μm for Nissl stain (0.5% cresyl echt violet acetate [Sigma Chemical, Oakville, ON, Canada]; which stains rRNA and ribosomes), and post-fixed in Zenker's solution (50% mercuric chloride, 25% potassium dichromate, 10% sodium sulphate, and 5% acetic acid; Sigma Chemical); or Kitoh-Matsushita silver stain for astrocytes (Kitoh and Matsushita 1980). Tissues were examined by light microscopy for pathology at $400\times$ and $1000\times$ magnification. Ten separate microscope fields were examined and the results combined as the raw score for each mouse. Statistical comparisons were made between groups of five mice for each treatment.

Confocal immunofluorescence microscopy

Because of the unusual early death of some mice exposed to the low intensity field (condition 6; 1–5 nT) compared to the higher intensity field (condition 5; 2–5 μT) in the double coil configuration, tissues from these mice were subjected to fluorescence immunocytochemistry.

Fluorescence confocal microscopy was performed for 6 μm sections from 15 tumours from mice exposed to the sham (condition 8), high intensity (condition 5), and low intensity (condition 6) fields

generated within the double coils. Confocal microscopy was used to more accurately reflect cell infiltration of the tissue sections (Biggerstaff et al. 2006). Sections were examined for leukocyte infiltration by staining with antibody-conjugates against the cluster of differentiation-4 (CD4), CD8, CD3, or CD45 markers (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The sections were deparrafinised in xylene, re-hydrated in an ethanol series, and heated in 0.01 M sodium citrate buffer to unmask antigens. The sections were blocked in 10% bovine serum albumin and 1% horse serum (InVitrogen, Burlington, ON, Canada) in phosphate-buffered saline (PBS), pH 7.4, and incubated for 1 h in appropriate primary antibody-fluorescein isothiocyanate (FITC) or phycoerythrin conjugates (Santa Cruz Biotechnologies) at 1:100 dilution in block buffer. The sections were analysed on a Zeiss LSM5 laser-scanning confocal microscope (Zeiss Canada, Toronto, ON) at 200× magnification with consistent laser settings. Digital images were obtained from multiple fields in the area of the tumour and the intensity of fluorescence in each image was ranked from 0 to 4: 0 = no immunoreactivity; 1 = mild; 2 = moderate; 3 = strong; and, 4 = very strong. Scoring was confirmed independently by a naïve observer. Statistical analysis among the different treatment groups was performed for each antibody. Ten separate microscope fields were examined and the results combined as the raw score for each mouse. Statistical comparisons were made between groups of five mice or each treatment.

Results

Overt tumours

There were conspicuous differences in the incidence and size of the B16-BL6 tumours among the different treatment groups 38 days after injection. The gross morphology of tumours in animals from different treatment groups are shown in Figure 2. The tumour mass was clearly demarcated from the adjacent subcutaneous tissue over the hindflank (beneath the injection area).

The number of mice in each treatment group (as described in Table I) that developed melanomas were: control (condition 1; 8/8); cylindrical field (condition 2; 7/8); Helmholtz coil (condition 3; 8/8); attenuated field (condition 4; 6/8); high intensity field (condition 5; 7/8); low intensity field (condition, 6; 5/5, 4 mice from this treatment group had died earlier); spatially rotating field (condition 7; 1/8); and, sham field-treated (condition 8; 8/8). There were no tumours present in 7 out of the 8 mice exposed to the spatially rotating field (Figure 2C). Chi-squared analysis demonstrated a significant difference [$\chi^2(7) = 33.09$, $p < 0.001$; $\varphi = 0.74$] in the presence of tumours among the groups. However, when the group exposed to the spatially rotating magnetic field was excluded there was no difference [$\chi^2(6) = 6.42$, $p > 0.05$; $\varphi = 0.34$].

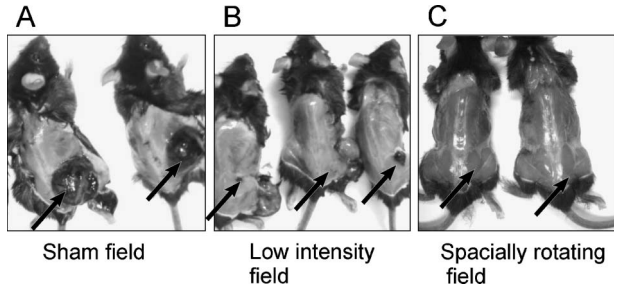

Figure 2. B16-BL6 tumour morphology in mice exposed to different spatial-temporal configurations of electromagnetic fields. C57 black mice were injected with B16-BL6 melanoma cells and subjected to a sham electromagnetic field condition (see Table I, condition 8) (Panel A), or to daily exposure for 3 h to a low intensity (1–5 nT) field (condition 6) (Panel B), or a rotating spatial temporal field of 1–5 μT (condition 7) (Panel C) for 38 days. An arrow marks the site of injection of the B16-BL6 cell suspension on day 1.

There were also differences in B16-BL6 tumour size among mice exposed to the different magnetic field configurations. In particular, the tumours were obviously smaller in the mice exposed nightly to the low intensity field of 1–5 nT (condition 6) generated by the double coils (Figure 2B). The mice exposed to the cylindrical field, Helmholtz field, attenuated field, high intensity field, or the sham field conditions formed large tumours that were not different from the non-handled controls (Figure 2A).

Treatment mortality

A significant proportion (4/9) of the mice treated with the magnetic field in the double coil, low intensity field (1–5 nT) geometry (condition 6) and injected with B16-BL6 tumour cells died within eight days of treatment. The animals that died were present in both experimental blocks (1/4 in block one and 3/5 in block two died) and none of the mice exposed to the other treatment conditions died during the 38 days of the experiment. The mice were randomised after injection of the B16-BL6 cells and those that died did not show significant or abnormal tumour growth (tumours are typically discernable two weeks after injection). It was noted that the mice showed 'stiff' movements for about two days prior to death.

Histology

The gross morphologies of all of the tumours that formed among the different treatment groups were

similar. In most cases, the tumours were surrounded by a pseudocapsule of connective tissue. Some tumour masses contained areas of fibrovascular stroma or loose lobulated connective tissue containing adipocytes. The melanoma cells formed sheets, clusters, or nest-like arrangements.

Foci of haemorrhage and different degrees of cell death, typical of large tumours, were also evident. The tumours present in the mice exposed to the different intensity fields generated in the double coil configuration (conditions 5 and 6) showed differences in a variety of histological features (Table II). For example, the number of mitotic indices, measured as the number of cells containing condensed heterochromatic figures in the cytoplasm per microscope field (mean and standard error for 10 microscopic fields), was significantly greater in the tumours of mice exposed to the sham (condition 8) and high intensity double coil field (condition 5) than in the tumours from mice exposed to the low intensity field (condition 6). Similarly, the presence of adipose tissue in the tumours, the number of mast cells, and the diameter of blood vessels within the tumour mass were greater in the tumours from mice exposed to the sham (condition 8) and high intensity fields (condition 5) than from mice exposed to the low intensity field (condition 6). The relative amount of monocyte/macrophage infiltration, measured as the ratio of extratumour monocyte/macrophages to intratumour monocyte/macrophages, was also smaller in the mice exposed to the low intensity field compared to mice exposed to the high intensity and sham fields.

Fluorescence immunohistochemistry

Leukocyte infiltration into the tumours of mice exposed to the sham (condition 8), high intensity field (condition 5), and the low intensity field (condition 6) were measured by fluorescence microscopy of tumour tissue stained with antibodies against T cell (CD3, CD4, and CD8) and panleukocyte (CD45) markers. The relative level of fluorescence was ranked (0–4; see *Methods*) for 10 microscopic fields and the mean and standard error for the fluorescence ratings corresponding to the presence of the leukocyte subtypes within the tumours are shown in Table III. These data show

Table II. Histological measures from melanoma tissue from mice exposed daily for 38 days to low (1–5 nT) or high intensity (3–5 μT) frequency-modulated magnetic fields.

	Sham		Low intensity		High intensity			
Variable	Mean	SE	Mean	SE	Mean	SE	F-value	eta
Mitotic index	17.7	2.9	8.3[a]	1.4	15.1	2.1	5.50	0.44
Adipose	1.0	0	0.4	0.2	1.0	0	5.49	0.43
Mast cells	38.3	14.2	10.6[a]	3.8	21.6	2.9	5.20	0.43
Monocyte ratio	1.3	0.2	0.7	0.1	1.4	0.2	6.37	0.48
Vascular size	10.0	2.5	6.7[a]	1.1	11.4	0.5	5.64	0.45

Data show the mean and standard error (SE) for 5 mice treated with the sham field (see Table I, condition 8), low intensity field (condition 6), or high intensity field (condition 5) for the number of mitotic figures and mast cells per microscopic field; the relative proportion of adipose tissue; the ratio of the number of monocytes present within the tumour tissue compared to the number present in adjacent non-tumour tissue; and, the measured diameter of any blood vessels found within the tumour. Ten microscopic fields were selected and evaluated as a raw score for each animal.
[a]indicates a significantly ($p < 0.05$) lower score compared to the sham field condition.

Table III. Fluorescence ratings for leukocyte subtypes within the melanoma tissue from mice exposed daily for 38 days to low (1–5 nT) or high intensity (3–5 μT) frequency-modulated magnetic fields.

	Sham		Low Intensity		High Intensity			
Measure	mean	SE	mean	SE	mean	SE	F-value	P
CD45	1.64	0.224	0.71[a]	0.140	1.20	0.359	5.315	0.008
CD3	2.59	0.215	2.14	0.173	2.30	0.153	1.528	0.227
CD4	2.00	0.363	2.30	0.40	1.85	0.337	0.403	0.671
CD8	1.29	0.322	0.80	0.359	0.54	0.268	1.582	0.220

Data show the mean and standard error (SE) for 5 mice treated with the sham field (see Table I, condition 8), low intensity field (condition 6), or high intensity field (condition 5) for the relative number of cells stained with the anti-CD45, anti-CD3, anti-CD4, and anti-CD8 antibodies per microscopic field. Ten microscopic fields were selected and evaluated as a raw score for each animal.
[a]indicates a significantly ($p < 0.05$) lower score compared to the sham field condition.

that the level of total leukocyte infiltration (CD45 + stained cells) was greater for tumours from mice exposed to the sham (condition 8) and high intensity fields (condition 5) than to the low intensity field (condition 6). However, these differences do not appear to be due to differences in T cell infiltration, since there were no significant differences in T cell infiltration between exposure treatments. In the tumours of mice exposed to the low intensity field, leukocyte and T cell staining was frequently associated with the inside of blood vessels but was not present in the extravascular tissues (Figure 3). On the other hand, the tumours of mice exposed to the sham and high intensity fields showed higher levels of leukocytes in the extravascular tissues and no conspicuous association with blood vessels.

Discussion

The results of this study support the hypothesis that specific temporal-spatial configurations of a weak (μT range) complex magnetic field pattern can inhibit the initial processes of cell proliferation by tumours. Mice exposed to a frequency-modulated magnetic field, which was rotated through the three spatial planes (condition 7), showed a strong suppression of tumour growth. Post-mortem inspection of these mice showed no evidence of visible tumour growth at the site of injection.

In these experiments, the mice were exposed to a spatially rotated magnetic field to determine the effects of changing the spatial and temporal configurations. Mice were also exposed to Helmholtz (condition 3) and double-coil magnetic field generating systems (conditions 4, 5 and 6) to determine if the effect was due only to the presence of the magnetic field. It was previously reported that homogenous fields, generated by Helmholtz coils, had a smaller effect on analgesia than exposure to heterogenous fields, generated by cylindrical fields, (Martin and Persinger 2003).

Therefore, we reasoned that maximum heterogeneity of the applied field through the exposure space over time should produce the strongest effect. A 3-D configuration (condition 7) was used where the frequency-modulated field (2–5 μT) was generated successively for 0.5 s through each pair of the opposing solenoids within the X, Y, and Z planes, and then all planes (X + Y + Z) simultaneously for 0.5 s (0.5 Hz) before the sequence began again.

The effect of magnetic fields on tumour growth has also been shown in other experimental models. For example, Yamaguchi et al. (2006) found that mice injected with similar melanoma cells and exposed for only 80 s/day for 17 days to a pulsed magnetic field was able to reduce the size of the tumour. However, they applied a field, whose strength was almost 100,000 more intense (0.25 T versus our 2–5 μT), directly against the tumour. Our

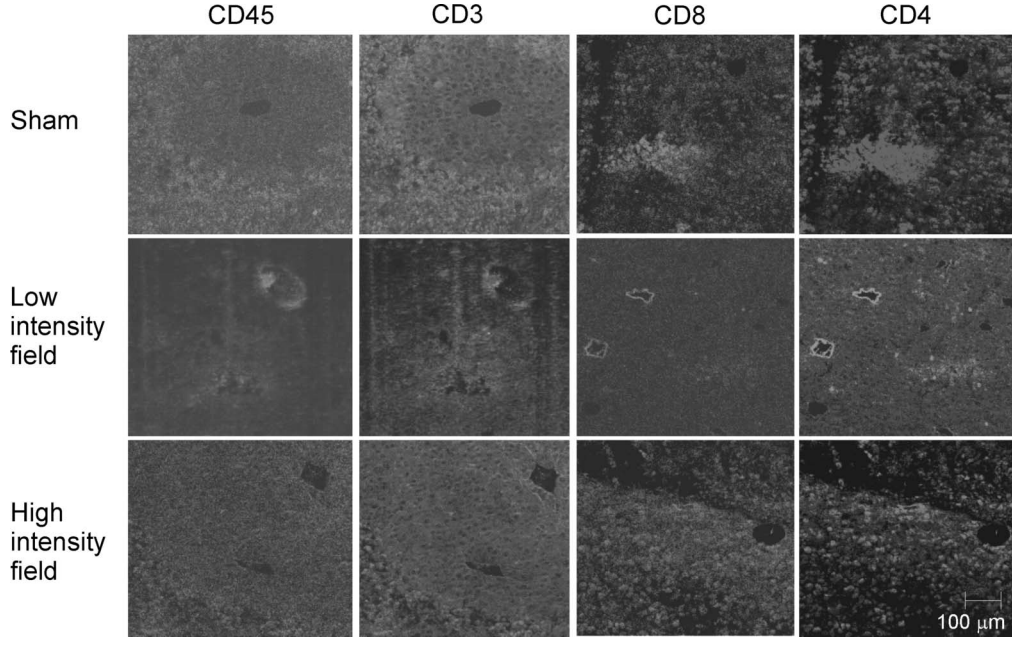

Figure 3. Fluorescence confocal microscopy results for CD45-, CD3-, CD8- and CD4-antigen positive cell localisation in tumours from mice exposed to sham (see Table I, condition 8), low intensity (condition 6), and high intensity (condition 5) electromagnetic fields. Tumour sections were stained with CD3 (FITC) and CD45 (phycoerythrin) or CD4 (FITC) and CD8 (phycoerythrin). Sections were visualised at 200 × magnification. The low intensity field condition contains brightly stained structures which correspond to leukocytes associated with blood vessels.

results suggest that whole body exposure to much weaker fields, well within the range, found within the cultural environment, might completely suppress the growth of these tumours if an appropriate waveform pattern of the field is used and if the direction of the applied fields are rotated successively through each of the spatial planes for 3 h nightly. We cannot exclude the possibility that the effectiveness in both studies might be related to the pulse frequency of about 25 Hz.

Except for moving the animal, our procedure was not invasive and did not require applying the field directly over the tumour. The procedure might be considered similar to exposing human beings while they work, study, or sleep within a room. Similar paradigms have been successful in reducing the development of experimental encephalomyelitis (Cook et al. 2000), the rodent model of multiple sclerosis. Whether or not the exposure during the scotophase, within red light, facilitated the effect must still be investigated empirically.

The results of this study also showed that the intensity of a specific magnetic field configuration generated in a single geometric plane can affect the survival of the mouse as well as the manner in which the tumour develops. About half of the mice exposed to the low intensity (1–5 nT) double coil configuration (condition 6) died within the first 8 days of treatment after cancer cell injection. Exposure to this field in the absence of injected tumour cells is not normally associated with increased mortality. In addition, we have injected over 200 mice with B16-BL6 cells over the last few years and such early mortality has not been seen in any other condition. The mice that died did not show any tumour development, which is typically only discernable after about two weeks. Within about two days of showing 'stiff' movements they were found dead. While the mechanisms underlying this affect are unknown, it may be related to the effects of magnetic fields on ion fluxes.

It is also possible that exposure to the field may sensitise the animals to neural dysfunction or inappropriate vascular/immunological events. Traditional light microscopy showed that the tumours of mice that had been exposed to the low intensity (1–5 nT) field (condition 6) and that remained alive, had fewer capillaries and fewer mast cells than those exposed to the high intensity (2–5 μT) fields (condition 5). The number of mitotic figures present in the tumour cells of mice exposed to the low intensity magnetic field was significantly lower that the number of mitotic figures seen in mice exposed to sham or high intensity (2–5 μT) fields. This suggests the possibility that low intensity magnetic fields are able to inhibit tumour cell division in the rapidly growing tumours.

The results of immunofluorescence microscopy experiments showed that the infiltration of leukocytes (CD45 antigen-positive cells) into the tumours was significantly different between the sham, low intensity, and high intensity magnetic fields. However, there were no significant differences in the infiltration of T cells, as determined by measuring the localisation of CD3, CD4, or CD8 positive T cell populations, in mice exposed to these experimental conditions.

This suggests that the difference in leukocyte infiltration does not reflect differences in T cell distribution but rather that alterations in other cell populations, such as natural killer cells, macrophages, or dendritic cells, may be responsible. In addition, the leukocytes in tissues from mice exposed to the low intensity fields (condition 6) were frequently associated with blood vessels rather than being distributed into the tumour tissues. This suggests the possibility that under those conditions the leukocytes are not able to extravasate and remain trapped in the blood vessels. This could be related to a potential failure in the formation of capillaries that allow tumour cell egress in the mice exposed to the low intensity fields. Such inhibition of capillary formation might also explain the smaller size of the tumours in this group. Further, if these treatment parameters promoted a relaxation in the physical integrity of the blood vessels and a loss in endothelial cell interactions, a systemic loss in vascular tone and decreased blood pressure might have occurred in the more vulnerable mice. Whether this mechanism could explain the premature deaths in four of the mice in this treatment group remains to be examined.

Many researchers have focused on experiments using highly homogeneous application geometries such as the Merritt and Helmholtz coils because of the ease of predicting the characteristics of the magnetic fields. In the present study, melanoma growth in mice exposed to the latter did not differ from controls. However, our results did show effects using coil-coil geometries and reiterate the usefulness of systematic investigation of the as yet unexplored varieties of spatialtemporal configurations of magnetic fields. The particular spatial-temporal configuration may be as important as the power density of electromagnetic field exposure (Brown and Chattopadhyay 1988) in order to understand the mechanisms by which these pervasive forces affect cancer.

Conclusion

The results of these studies showed that exposure to a specific temporal-spatial configuration of a weak, complex magnetic field could inhibit the growth of experimental tumours. These effects were also

dependent on the intensity and special complexity of the pattern. The most potent inhibition of tumour growth was in mice exposed to the magnetic field rotated successively through the three spatial dimensions. However, exposure to a heterogenous low intensity (1–5 nT) field generated between double coils also significantly inhibited tumour growth while exposure to a high intensity (2–5 μT) field was not different from sham conditions.

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Note

A recent report by V.V. Novikov, G.V. Novikov, and E.E. Fesenko (2009) [Effect of weak combined static and extremely low-frequency alternating magnetic fields on tumor growth in mice inoculated with the Ehrlich ascites carcinoma. Bioelectromagnetics 30:343] has described potent anti-tumor effects of low intensity (100–300 μT), low frequency (1–16.5 Hz) alternating magnetic fields on mice transplanted with the Ehrlich carcinoma. The conclusions from this study are in support of what we have reported using a different model system.

References

Alfieri RR, Bonelli MA, Pedrazzi G, Desenzani S, Ghillani M, Fumarola C, Ghibelli L, Borghetti AF, Petronini PG. 2006. Increased levels of inducible HSP70 in cells exposed to electromagnetic fields. Radiation Research 165:95–104.

Antonini RA, Benfante R, Gotti C, Moretti M, Kuster N, Schuderer J, Clementi F, Fornasari D. 2006. Extremely low frequency electromagnetic field (ELF-EMF) does not affect the expression of alpha3, alpha5, and alpha7 nicotinic receptor subunit genes in SH-SY5Y neuroblastoma cell line. Toxicology Letters 164:268–277.

Bernardini C, Zannoni A, Turba ME, Bacci ML, Forni M, Mesirca P, Remondini D, Catellani G, Bersani F. 2007. Effects of 50 Hz sinusoidal magnetic fields on Hsp27, Hsp70, Hsp90 expression in porcine aortic endothelial cells (PAEC). Bioelectromagnetics 28:231–237.

Biggerstaff J, Weidow B, Amirkhosravi A, Francis JL. 2006. Enumeration of leukocyte infiltration in solid tumours by confocal laser scanning microscopy. BMC Immunology 7:16.

Blackman CF, Benane SG, House DE, Elliott DJ. 1990. Importance of alignment between local DC magnetic field and an oscillating magnetic field in response of brain tissue in vivo and in vitro. Biolectromagnetics 11:159–167.

Blanchard JP, Blackman CF. 1994. Clarification and application of an ion parametric resonance model for magnetic field interactions with biological systems. Bioelectromagnetics 15: 217–238.

Brown HD, Chattopadhyay SK. 1988. Electromagnetic-field exposure and cancer. Cancer Biochemistry and Biophysics 9: 295–342.

Cook LL, Persinger MA, Koren SA. 2000. Differential effects of low frequency, low intensity (<6 mG) nocturnal magnetic fields upon infiltration of mononuclear cells and numbers of mast cells in Lewis rat brains. Toxicology Letters 118:9–19.

Coulton LA, Harris PA, Barker AT, Pockley AG. 2004. Effect of 50 Hz electromagnetic fields on the induction of heat-shock protein gene expression in human leukocytes. Radiation Research 161:430–434.

George I, Geddis MS, Lill Z, Lin H, Gomez T, Blank M, Oz MC, Goodman R. 2008. Myocardial function improved by electromagnetic field induction of stress protein hsp70. Journal of Cellular Physiology 216:816–823.

Girgert R, Grundker C, Emons G, Hanf V. 2008. Electromagnetic fields alter the expression of estrogen receptor cofactors in breast cancer cells. Bioelectromagnetics 29:169–176.

Ishido M, Nitta H, Kabuto M. 2001. Magnetic fields (MF) of 50 Hz at 1.2 microT as well as 100 microT cause uncoupling of inhibitory pathways of adenylyl cyclase mediated by melatonin 1a receptor in MF-sensitive MCF-7 cells. Carcinogenesis 22:1043–1048.

Jin M, Blank M, Goodman R. 2000. ERK1/2 phosphorylation, induced by electromagnetic fields, diminishes during neoplastic transformation. Journal of Cellular Biochemistry 78: 371–379.

Kaszuba-Zwoinska J, Gil K, Ziomber A, Zaraska W, Pawlicki R, Krolczyk G, Matyja A, Thor PJ. 2005. Loss of interstitial cells of Cajal after pulsating electromagnetic field (PEMF) in gastrointestinal tract of the rats. Journal of Physiology and Pharmacology 56:421–432.

Kaszuba-Zwoinska J, Ciecko-Michalska I, Madroszkiewicz D, Mach T, Slodowsk-Hajduk Z, Rokita E, Zaraska W, Thor P. 2008. Magnetic field anti-inflammatory effects in Crohn's disease depends upon viability and cytokine profile of the immune competent cells. Journal of Physiology and Pharmacology 59:177–187.

Keklikci U, Akpolat V, Ozekinci S, Unlu K, Celik MS. 2008. The effect of extremely low frequency magnetic field on the conjunctiva and goblet cells. Current Eye Research 33: 441–446.

Kitoh T, Matsushita M. 1980. A new staining method of astrocytes for paraffin section. Acta Neuropathologica (Berlin) 49:67–69.

Lang PG. 2002. Current concepts in the management of patients with melanoma. American Journal of Clinical Dermatology 3: 401–426.

Lednev VV. 1991. Possible mechanisms for the influence of weak magnetic fields on biological systems. Bioelectromagnetics 12: 71–75.

Li JK, Lin JC, Liu HC, Chang WH. 2007. Cytokine release from osteoblasts in response to different intensities of pulsed electromagnetic stimulation. Electromagnetic Biology and Medicine 26:153–165.

Liboff AR, Rozek RJ, Sherman ML, McLeod BR, Smith SD. 1987. Ca^{45} cyclotron resonance in human lymphocytes. Journal of Bioelectricity 6:13–32.

Lin H, Blank M, Rossol-Haseroth K, Goodman R. 2001. Regulating genes with electromagnetic response elements. Journal of Cellular Biochemistry 81:143–148.

Lisi A, Foletti A, Ledda M, Rosola E, Giuliani L, D'Emilia E, Grimaldi S. 2006. Extremely low frequency 7 Hz 100 microT electromagnetic radiation promotes differentiation in the human epithelial cell line HaCaT. Electromagnetic Biology and Medicine 25:269–280.

Martin LJ, Persinger MA. 2003. Spatial heterogeneity not homogeneity of the magnetic field during exposures to complex frequency-modulated patterns facilitates analgesia. Perception and Motor Skills 96:1005–1012.

Martin LJ, Koren SA, Persinger MA. 2004. Thermal analgesic effects from weak, complex magnetic fields and

pharmacological interactions. Pharmacology Biochemistry and Behavior 78:217–227.

McKay BE, Persinger MA, Koren SA. 2000. Exposure to a theta-burst patterned magnetic field impairs memory acquisition and consolidation for contextual but not discrete conditioned fear in rats. Neuroscience Letters 292:99–102.

Nicolin V, Ponti C, Baldini G, Gibellini D, Bortul R, Zweyer M, Martinelli B, Narducci P. 2007. In vitro exposure of human chondrocytes to pulsed electromagnetic fields. European Journal of Histochemistry 51:203–212.

Nie K, Henderson A. 2003. MAP kinase activation in cells exposed to 60 Hz electromagnetic field. Journal of Cellular Biochemistry 90:1197–1206.

Olcese JM. 1990. The neurobiology of magnetic field detection in rodents. Progress in Neurobiology 35:325–330.

Persinger MA, Belanger-Chellew G. 1999. Facilitation of seizures in limbic epileptic rats by complex 1 microTesla magnetic fields. Perception and Motor Skills 89:486–492.

Persinger MA. 2003. Neurobehavioral effects of brief exposures to weak intensity, complex magnetic fields within experimental and clinical settings. In McLean MJ, Engstrom S, Holocomb RR, editors. Magnetotherapy: Potential therapeutic benefits and adverse effects. New York: TFG Press. pp 89–118.

Persinger MA, McKay BE, O'Donovan CA, Koren SA. 2005. Sudden death in epileptic rats exposed to nocturnal magnetic fields that simulate the shape and intensity of sudden changes in geomagnetic activity: An experimental response to Schnabl, Beblo and May. International Journal of Biometeorology 49: 256–261.

Pilla AA, Muehsam DJ, Markov MS, Sisken BF. 1999. EMF signals and ion/ligand binding kinetics: Prediction of bioeffective waveform parameters. Bioelectrochemistry and Bioenergetics 48:27–34.

Reale M, De Lutiis MA, Patruno A, Speranza L, Felaco M, Grilli A, Macri MA, Comani S, Conti P Di Luzio S. 2006. Modulation of MCP-1 and iNOS by 50-Hz sinusoidal electromagnetic field. Nitric Oxide 15:50–57.

Simko M, Mattsson MO. 2004. Extremely low frequency electromagnetic fields as effectors of cellular responses in vitro: Possible immune cell activation. Journal of Cellular Biochemistry 93:83–92.

St-Pierre LS, Persinger MA, Koren SA. 1998. Experimental induction of intermale aggressive behavior in limbic epileptic rats by weak, complex magnetic fields: Implications for geomagnetic activity and the modern habitat? International Journal of Neuroscience 96:149–159.

Thomas AW, Kavaliers M, Prato FS, Ossenkopp KP. 1997. Antinociceptive effects of a pulsed magnetic field in the land snail, *Cepaea nemoralis*. Neuroscience Letters 222: 107–110.

Vianale G, Reale M, Amerio P, Stefanachi M, Di Luzio S, Muraro R. 2008. Extremely low frequency electromagnetic field enhances human keratinocyte cell growth and decreases proinflammatory chemokine production. British Journal of Dermatology 158:1189–1196.

Walther M, Mayer F, Kafka W, Schutze N. 2007. Effects of weak, low-frequency pulsed electromagnetic fields (BEMER type) on gene expression of human mesenchymal stem cells and chondrocytes: An in vitro study. Electromagnetic Biology and Medicine 26:179–190.

Yamaguchi S, Ogiue-Ikeda M, Sekino M, Ueno S. 2006. Effects of pulsed magnetic stimulation on tumor development and immune functions in mice. Bioelectromagnetics 27:64–72.

Zhang X, Zhang J, Qu X, Wen J. 2007. Effects of different extremely low-frequency electromagnetic fields on osteoblasts. Electromagnetic Biology and Medicine 26:167–177.